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(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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[Continued on next page]

(54) Title: MUTANT E. COLI APPA PHYTASE ENZYMES AND NATURAL VARIANTS THEREOF, NUCLEIC ACIDS ENCODING SUCH PHYTASE ENZYMES, VECTORS AND HOST CELLS INCORPORATING SAME AND METHODS OF MAKING AND USING SAME

Random mutagenesis cycle 1 (starting material: clone 18B2)

Library #1 (size: 10,000)	clones screened	selected hits
	8,813	41
	5,118	29
	3,075(F)	20(F)
	25,035	29

Library #2

Random mutagenesis cycle 2

Library #2 (size: 29,300)	clones screened	selected hits
	19,491	49
	9,946	50
		50

Library #3A

Library #4A

rescreen pool	selected hits
5606	9 (#735-743)

Library #5A

Random mutagenesis cycle 3

Library #3A (size: 20,000)	clones screened	selected hits
	8,488	50 (#635-#684)

Library #4A (size: 30,000)	clones screened	selected hits	rescreen pool	selected hits
	21,564	50 (#685-#734)	2134	15 (#844-#858)
	21,100	50 (#744-#793)		

Library #5A (size: 100,000)	clones screened	selected hits
	21,763	50 (#794-#843)
	30,921	50 (#859-#908)

(57) Abstract: Cells producing mutant phytases having modified activity are provided, as well as the phytases so produced. Also provided are methods of making and producing such phytases and the use of the expressed phytase protein in feed as a supplement.



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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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(57) Abstract: Cells producing mutant phytases having modified activity are provided, as well as the phytases so produced. Also provided are methods of making and producing such phytases and the use of the expressed phytase protein in feed as a supplement.

WO 2004/015084 A3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/25058

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 9/16, 15/55
US CL : 435/196; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/196; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RODRIGUEZ, E. et al. Site-Directed Mutagenesis Improves Catalytic Efficiency and Thermostability of Escherichia coli pH 2.5 Acid Phosphatase/Phytase Expressed in Pichia pastoris. 01 October 2000, Vol. 382, No. 1, pages 105-112, see particularly Fig. 1	1-3
X,P	US 2002/0136754 A1 (SHORT et al.) 26 September 2002 (26.09.2002), see particularly Figure 8.	1-3
Y	LIM, D. et al. Crystal Structures of Escherichia coli Phytase and Its Complex With Phytate. Nature Structural Biology, February 2000, Vol. 7, No. 2, pages 108-113, see particularly pages 110-111 and Fig 3	1-3,

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

<p>* Special categories of cited documents:</p>	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

23 July 2004 (23.07.2004)

Date of mailing of the international search report

10 SEP 2004

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INTERNATIONAL SEARCH REPORT

Continuation of Item 4 of the first sheet:

existing title is too long

NewTitle: Mutant *E. coli* AppA Phytase Enzymes**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING**

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-3, drawn to a modified phytase having a modification of a residue within 5 amino acids and/or 6 Å of the alpha carbon of residue 26 of the mature *E. coli* phytase designated EBC18B2, nucleic acids, vectors and host cells encoding said modified phytases and methods of making said phytases.

Group II, claim(s) 1-3, drawn to a modified phytase having a modification of a residue within 5 amino acids and/or 6 Å of the alpha carbon of residue 43 of the mature *E. coli* phytase designated EBC18B2, nucleic acids, vectors and host cells encoding said modified phytases and methods of making said phytases.

Group III, claim(s) 1-3, drawn to a modified phytase having a modification of a residue within 5 amino acids and/or 6 Å of the alpha carbon of residue 46 of the mature *E. coli* phytase designated EBC18B2, nucleic acids, vectors and host cells encoding said modified phytases and methods of making said phytases.

Group IV, claim(s) 1-3, drawn to a modified phytase having a modification of a residue within 5 amino acids and/or 6 Å of the alpha carbon of residue 54 of the mature *E. coli* phytase designated EBC18B2, nucleic acids, vectors and host cells encoding said modified phytases and methods of making said phytases.

Group V, claim(s) 1-3, drawn to a modified phytase having a modification of a residue within 5 amino acids and/or 6 Å of the alpha carbon of residue 73 of the mature *E. coli* phytase designated EBC18B2, nucleic acids, vectors and host cells encoding said modified phytases and methods of making said phytases.

Group VI, claim(s) 1-3, and 16-18, drawn to a modified phytase having a modification of a residue within 5 amino acids and/or 6 Å of the alpha carbon of residue 113 of the mature *E. coli* phytase designated EBC18B2, nucleic acids, vectors and host cells encoding said modified phytases and methods of making said phytases.

Group VII, claim(s) 1-3, drawn to a modified phytase having a modification of a residue within 5 amino acids and/or 6 Å of the alpha carbon of residue 126 of the mature *E. coli* phytase designated EBC18B2, nucleic acids, vectors and host cells encoding said modified phytases and methods of making said phytases.

Group VIII, claim(s) 1-3, drawn to a modified phytase having a modification of a residue within 5 amino acids and/or 6 Å of the alpha carbon of residue 184 of the mature *E. coli* phytase designated EBC18B2, nucleic acids, vectors and host cells encoding said modified phytases and methods of making said phytases.

Group IX, claim(s) 1-3, drawn to a modified phytase having a modification of a residue within 5 amino acids and/or 6 Å of the alpha carbon of residue 228 of the mature *E. coli* phytase designated EBC18B2, nucleic acids, vectors and host cells encoding said modified phytases and methods of making said phytases.

Group X, claim(s) 1-3, drawn to a modified phytase having a modification of a residue within 5 amino acids and/or 6 Å of the alpha carbon of residue 384 of the mature *E. coli* phytase designated EBC18B2, nucleic acids, vectors and host cells encoding said modified phytases and methods of making said phytases.

Group XI, claim(s) 1-3, drawn to a modified phytase having a modification of a residue within 5 amino acids and/or 6 Å of the alpha carbon of residue 410 of the mature *E. coli* phytase designated EBC18B2, nucleic acids, vectors and host cells encoding said modified phytases and methods of making said phytases.

Group XII, claim(s) 13-15 and 19, drawn to a methods of modifying a phytase.

The inventions listed as Groups I-XII do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The only shared technical feature of Groups I-XII is that they all related to a modified phytase having a modification within 5 residues and/or 6 Å of the alpha carbon of

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/25058

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claim Nos.: 4--12
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-3 as drawn to phytase with modification of residue 26

Remark on Protest

☐
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

residues 26, 43, 46, 54, 73, 113, 126, 184, 228, 384, or 410 of the mature *E.coli* phytase designated EBC18B2. However, this shared technical feature is not a special technical feature as defined by PCT Rule 13.2 as it does not define a contribution over the art. Rodriguez et al. teach modified phytases having mutations at positions A131, V134, D207, and S211 (which correspond to positions 109, 112, 185, and 189, respectively, of the mature *E.coli* phytase designated EBC18B2). Thus the only shared technical feature of Groups I-XII is not a special technical feature as defined by PCT Rule 13.2.

Continuation of B. FIELDS SEARCHED Item 3:

EAST, MEDLINE, SCISEARCH, LIFESCI, BIOTECHDS, BIOSIS, EMBASE, CA, NTIS, ESBIODASE, BIOTECHNO, WPI search terms: phytase#, AppA, muta?, variant#